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An impedance spectroscopy method for the detection and evaluation of *Babesia bovis* antibodies in cattle

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ABSTRACT

An immunosensor method for diagnosis of *Babesia bovis* in cattle based on impedance measurement is presented in this study. The method probes the interaction between antibodies present in serum of *B. bovis* infected cattle and a recombinant version of the C-terminal portion of RAP-1 obtained from the Portuguese *B. bovis* Santarém strain (rRAP-1/CT-STR). Following immobilization of rRAP-1/CT-STR on gold electrodes through the formation of a self-assembled layer, the alteration of the interface properties was traced by electrochemical impedance spectroscopy (EIS). The changes of the impedimetric properties of the deposited rRAP-1/CT-STR *B. bovis* protein layer, interaction with different concentrations of anti-rRAP-1/CT-STR antibodies, and the association constants of the immunoreactive molecules involved, were obtained using EIS. The results were compared with an enzyme-linked immunosorbent assay using the same antigen, and immunofluorescence. The results confirmed the potential for further developing of an immunosensor-based method for the detection and characterization of antibodies against *B. bovis* in cattle. In addition, such method would provide the advantage of determining the association constant between antibody–antigen interactions without the use of labelling molecules.

1. Introduction

Babesiosis, is an acute parasitic disease caused by hemoprotozoan apicomplexan parasites from the genus *Babesia* and is transmitted by ticks in the Ixodidae family [1]. Bovine babesiosis is an economically important tick-borne disease of cattle in tropical and subtropical regions of the world. The clinical signs developed as a result of *Babesia bovis* are characterized by fever, anemia, and icterus in the infected cattle. Acute infections are usually diagnosed by microscopic examination of blood smears, whereas subclinical chronic infections can be identified serologically [2]. In addition, the indirect fluorescent antibody test (IFAT) is one of the most commonly used methods for the diagnosis of *B. bovis* infection [3,4] and has been applied in epidemiological studies in a number of countries [5,6], but IFAT was rendered cumbersome and subjective [4,7].

The well-conserved *B. bovis* rhoptry-associated protein 1 (RAP-1) gene encoding a 60 kDa merozoite apical membrane polypeptide was previously identified and characterized [8,9]. The major immunogenic B-cell and T-cell epitopes on RAP-1 are conserved among all strains tested, but they are not conserved between different species [10,11]. Importantly, the C-terminus region of RAP-1 contains geographically conserved B-cell epitopes that can be helpful in the development of species-specific diagnostic assays [12]. Because of its immunogenic properties and the lack of extensive differences in RAP-1 among geographically distinct isolates of *B. bovis*, RAP-1 was extensively used as a diagnostic tool. Thus, an antigen derived from the C-terminal region of RAP-1 was used for the development and validation of a competitive ELISA [13–15].

Immunosensors have become simple and automated tools for monitoring immunochemical substances, with high sensitivity and selectivity, successfully applied in clinical and pharmaceutical analysis as well as for determination of contaminants in environmental monitoring [16]. The aim of this study is the examination of the electrochemical impedance spectroscopy (EIS) properties of the thiol-acid monolayer and recombinant RAP-1/CT *B. bovis* protein layer on gold electrode surface as an immunosensor tool.

Impedance measurement of immunoreactions has been considered a plausible alternative method for immunological assays [17]. Depending on the configuration adopted, information about the antigen–antibody interaction, including evaluation of the association constant, may be obtained from capacitance or resistance. The

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characterization of biomaterial interface features and the data provided by the ELISA assays are combined in a way that allows a better understanding of the surface properties in regard to the biosensor sensitivity and reproducibility. In this study, an open reading frame encoding for the RAP-1/CT *B. bovis* protein obtained from Portuguese strains isolated from Santarém (STR), a region located in the Central region of Portugal, was expressed in *E. coli*, purified and immobilized onto a impedimetric sensor gold layer that was then used to measure interaction with antibody from recombinant *B. bovis* RAP-1/CT immunized mice, and from cattle sera samples by EIS.

2. Materials and methods

2.1. Parasites

The Mo7 biological clone of *B. bovis* was derived by limiting dilution of the Mexico strain as described [18,19] and was maintained as a cryopreserved stabilate in liquid nitrogen [20]. Parasites were grown in long term microaerophilous stationary-phase culture by previously described techniques [19,21]. The Santarém strain of *B. bovis* was isolated from blood collected from field animal at the Santarém region located in Central Portugal.

2.2. Reagents and solutions

All chemicals and solvents used throughout this study were of analytical grade Milli-Q filtered water quality and the prepared solutions were kept at 4 °C before use. The 16-mercaptoundecanoic acid, 1-ethyl-3(3-dimethyl aminopropyl)carbodiimide (EDC), *N*-hydroxy succinimide (NHS), hydrogen peroxide (H_2O_2), sulphuric acid (H_2SO_4), potassium ferrocyanide(II) trihydrate reagent, potassium ferricyanide(III) reagent, L-arabinose, 5-bromo-4-chloro-3-indolyl-_-D-galactopyranoside (X-Gal) 3,5, 3′,5′-tetramethylbenzidine (TMB), bovine anti-rabbit immunoglubin G (IgG), anti-mouse IgG conjugated with alkaline phosphatase, fluoresceinated isothiocyanate-conjugated goat anti-mouse IgG and fluoresceinated isothiocyanate-conjugated rabbit anti-bovine IgG were obtained from Sigma–Aldrich, and horseradish peroxidase-labeled mouse anti-bovine IgG was obtained from Santa Cruz Biotechnology Inc. Miller's Luria broth medium, ampicillin and ProBond resin column were obtained from Invitrogen. ELISA plates were obtained from Nunc (Immuno Plate MaxiSorp™). The mouse polyclonal antibody against recombinant RAP-1/CT-STR protein of *B. bovis* (anti-rRAP-1/CT-STR antibody) was produced by Biotechnology Department, INETI, Portugal. The ability of the antibodies against the rRAP-1/CT-STR antigen to react with native RAP-1 from the Mo7 strain of *B. bovis* was determined by ELISA, immunofluorescence and Western blot analysis [14,22,23].

The following buffers were used in this study: carbonate buffer: 15 mM Na_2CO_3; 35 mM Na_2HCO_3, pH 9.6; phosphate buffered solution (PBS): NaCl 0.150 M, KCl 0.027 M, Na_2HPO_4 0.570 M, KH_2PO_4 0.003 M, pH 7.4; PBS redox: $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, 5 mM; wash buffer: 0.05% Tween 20 in PBS; EIS blocking buffer: 0.2% casein in PBS; ELISA blocking buffer: 20% skim milk in PBS; ELISA dilution buffer: 0.1% BSA in PBS, ELISA stop buffer: H_2SO_4 2 M.

2.3. Cloning, expression, and purification, of rRAP-1/CT-STR from Portuguese B. bovis strains

The rRAP-1/CT-STR *B. bovis* DNA coding region was amplified by PCR from genomic DNA extracted from the blood of field isolate *B. bovis*-infected Portuguese cattle. The DNA was extracted using a FlexiGene DNA kit (Quiagen). The primers used were *RAP-1/CT forward* (5′-CCT ACA AAG AAG TTC ATC GAG G-3′); *RAP1–TOPO reverse* (5′-GAG GTA TCC GGC GGT GTG TTC AC-3′) [14]. The 747 bp PCR amplicon was cloned into pBAD/TOPO ThioFusion (Invitrogen), transformed into *E. coli* cells and selected on Miller's Luria broth with 50 μg/ml ampicillin (LBA) plates containing X-Gal. The white colonies containing inserts were picked and screened with *TrX forward* (5′-TTC CTC GAC GCT AAC CTG-3′) (Invitrogen) and *RAP1–TOPO reverse*. Colonies with PCR products of the correct size were grown overnight in LBA media, and the plasmid isolated using a miniprep kit (Wizard-Plus SV; Promega Inc.). The insert orientation and frame of each clone was confirmed by sequencing in both orientations using primers *TrX forward* and *pBAD reverse* (5′-GAT TTA ATC TGT ATC AGG-3′) (Invitrogen). Sequencing was performed with the Big Dye version 3.1 Cycle Sequencing Kit from Applied Biosystems with an ABI 3730 DNA Analyzer.

For expression of rRAP-1/CT-STR, single recombinant *E. coli* colonies were inoculated into LBA media and incubated overnight at 37 °C with shaking. Protein expression was induced with 0.01% L-arabinose for 4 h. *E. coli* were centrifuged, lysed, and sonicated. The recombinant protein was then purified on a ProBond resin column as suggested by the manufacturer (Invitrogen). The purified recombinant protein yield was determined by spectrophotometry and the purification and specificity of the antigen was analysed by pre-cast SDS-polyacrylamide gel electrophoresis 4–12% (Bio-Rad) with Coomassie Blue staining and Western blotting, respectively, as described by Goff et al. [14]. Protein concentration was measured by absorbance readings at 280 nm using a spectrophotometer.

2.4. Processing of bovine sera

Cattle blood samples were collected from Portuguese field animals under sterile conditions, and transported refrigerated to the laboratory. Serum was obtained from the blood samples collected without anticoagulants by centrifugation for 20 min at 420 × *g* and 4 °C, and then stored at −20 °C. In order to minimize non-specific reactivity of the sera with *E. coli* antigen present in the coated recombinant antigen, serum samples were incubated for 1 h at 37 °C with 50 μg/ml of *E. coli* lysate. After centrifugation at 9300 × *g* for 15 min, the supernatant was collected and used in immunoassays. Lysate was prepared from *E. coli* TOP10 (Chemically Competent) strain transformed with the pBAD/TOPO ThioFusion vector and induced with 0.01% L-arabinose.

2.5. Fixed immunofluorescence antibody test (IFAT)

Fixed IFAT was performed as described previously by Wilkowsky et al. [23] and de Rios et al. [22] to test the reactivity of anti-rRAP-1/CT-STR antibody with fixed *B. bovis* Mo7 infected red blood cells. Fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG and FITC rabbit anti-bovine IgG were used as secondary antibodies for mouse and bovine host, respectively. Slides were observed under fluorescence microscopy. Each of the slides contained 15 wells, with 3 of them used for controls. A positive control, *B. bovis* infected serum, and two negative controls, consisting either of a non-infected serum or the secondary FITC antibody on fixed *B. bovis* Mo7 infected red blood cells were also performed.

2.6. Indirect ELISA

Indirect ELISA was adapted from Boonchit et al. [2]. rRAP-1/CT-STR *B. bovis* purified recombinant protein (0.002 μg/μl) diluted in carbonate buffer pH 9.6 was added to each well of the plate and incubated overnight at 4 °C. rRAP-1/CT-STR-coated plates were blocked with 200 μl of ELISA blocking buffer for 2 h at 37 °C. After the blocking buffer was aspirated off, the plate was washed one time in wash buffer. After the washing, 100 μl of diluted serum

(1:40 and 1:80) in PBST was added to each well (including positive and negative control sera as described above), and the plate was incubated at room temperature for 50 min. The plate was then washed three times with 200 μl of wash buffer, followed by the addition of 100 μl of horseradish peroxidase-labeled mouse anti-bovine IgG (diluted 1:1000 in PBST) to each well. After incubation at room temperature for 55 min, each plate was washed six times in 200 μl of wash buffer as before, and then allowed to incubate for 60 s in wash buffer, then washed again three times in wash buffer. 100 μl of TMB substrate was added to each well and the plates were then incubated in the dark at room temperature for 30 min, followed by the addition of 75 μl of stop buffer. The mean optical density (OD) at 450 nm was determined for all test wells using a microtiter plate reader (Spectra max 340—Molecular Devices).

2.7. Concentration of polyclonal antibody by ELISA

To estimate the concentration of polyclonal antibody used in Section 3.2.2. We developed an ELISA using monoclonal antibody BABB75 that binds an epitope in the C-terminal region of *B. bovis* RAP-1 [10]. A 96-well plate containing rRAP-1/CT-STR *B. bovis* (0.002 μg/μl) prepared as described above, was incubated with known amounts of the monoclonal antibody BABB75 at 1:2 dilutions (starting in 1:2–1:256) and absorbance (450 nm) was measured after incubation with peroxidase-labelled secondary anti-mouse antibody. Results were plotted and the slope of the curve calculated using the equation $y = ax + b$. The slope was then used to calculate the concentrations of polyclonal antibody anti rRAP-1/CT-STR, based on their absorbance's at 450 nm.

2.8. Electrochemical impedance spectroscopy

The electrochemical behaviour of the immunoreaction layer on the biosensor gold coat was investigated by measuring the impedance. The experiments were carried out either in aqueous solutions using a conventional three-electrode system with the modified or bare gold substrate as the working electrode (surface 0.11 cm^2), a platinum plate as the auxiliary electrode (surface 0.54 cm^2), and a saturated calomel reference electrode (SCE).

The impedance measurements were performed using a Voltalab 40 impedance analyser (Radiometer Analytical SA, Villeurbanne, France). An equimolar ferrocyanide/ferricyanide mixture (5 mM) in PBS solution (pH 7) was used. Impedance measurements were recorded by frequencies ranging from 100 kHz to 50 mHz, with an AC of 10 mV peak to peak amplitude, with 5 points per decade of frequencies. The typical biosensor impedance responses were recorded at 0.2 V applied potential in order to avoid the likelihood of faradaic process. Faradaic process can occur on the surface of naked gold electrode with the redox species under analysis, as described previously by Tlili et al. [24]. Immunoreactions were performed in an electrochemical cell containing 5 ml of PBS redox buffer at room temperature. All potentials were measured with respect to the SCE. The Zview software (Scribner Associates) was used for complex circuit modelling.

2.9. Gold preparation and antibodies immobilization

2.9.1. Gold cleaning

Gold electrode preparation and antibody immobilization were performed as described previously by Tlili et al. [24]. The gold electrodes were cleaned with organic solvents (acetone and ethanol) and with piranha solution (3:7 H_2O_2-concentrated H_2SO_4) for 1 min, to obtain a clean gold surface. After each pre-treatment, the gold substrates were rinsed successively with Milli-Q filtered water and ethanol and than dried with a stream of N_2.

2.9.2. Self-assembled monolayer

The pre-treated electrodes were immersed in an ethanol solution of 1 mM 11-mercaptoundecanoic acid for 12 h to obtain a self-assembled monolayer (SAM). The gold substrates were then rinsed with ethanol in order to remove the non-bonded thiol. To convert the terminal carboxylic groups of the arrayed acids to active NHS esters, the thiol-modified electrodes were treated with 0.4 mM EDC–0.1 mM NHS for 1 h.

2.9.3. Immobilization of antigen

After rinsing the gold electrodes with water, 0.1 mg/ml of rRAP-1/CT-STR *B. bovis* protein was dropped onto the surface and incubated at 37 °C for 90 min. The excess antigen was removed by rinsing with PBS, and PBS redox was added for impedance measurement. The protein-modified electrodes were then treated with 0.2% casein for 1 h to block the non-specific sites. The excess protein was removed by rinsing with PBS, and later PBS redox was added for impedance measurement. After this preparation of the surface, different concentrations of anti-rRAP-1/CT-STR *B. bovis* antibody and sera from field cattle were used for measurements.

3. Results and discussion

3.1. Identification and characterization of rRAP-1/CT-STR from Portugal strain of B. bovis

RAP-1/CT was PCR-amplified from the Portuguese *B. bovis* STR strain isolated from Santarém, a region located in the Central region of Portugal. Sequence comparison of the RAP-1/CT gene from the published strain *B. bovis* T2Bo and the STR strains show 99% of identity (data not shown). RAP-1/CT gene from the STR strain was cloned and expressed in the pBAD/TOPO ThioFusion vector, and purified for subsequent studies. Purified rRAP-1/CT-STR protein reacts with the monoclonal antibody BABB75, that recognize a conserved and repetitive RAP-1 B-cell epitope present in the C-terminus of the molecule in immunoblots and ELISA (data not shown) [14].

A mouse polyclonal anti-rRAP-1/CT-STR antibody was produced and its reactivity tested against the *B. bovis* Mo7 strain by IFAT and western blot. Fig. 1A shows the results obtained by IFAT method, where the fluorescence of intraerythrocytic parasites reacting with the anti-rRAP-1/CT-STR antibody with the punctuate pattern typical for *B. bovis* RAP-1 [15] can be observed. In contrast, non-infected erythrocytes do not react with the anti-rRAP-1/CT-STR antibody. In addition, mouse anti-rRAP-1/CT-STR antibody specifically reacted with a single antigen present in infected erythrocytes lysates of the *B. bovis* Mo7 strain with molecular weight of approximately 60 kDa in western blots. The size of the band is consistent with the expected molecular weight of native RAP-1 protein [15]. In addition, the mouse polyclonal anti-rRAP-1/CT-STR antibody is able to react specifically with rRAP-1/CT-STR in immunoblots (data not shown). In conclusion, the IFAT and western blot results suggests that the mouse anti-rRAP-1/CT-STR antibody reacts against both, native RAP-1, and the recombinant rRAP-1/CT-STR protein. These immunoreagents will be used for the development of an electrochemical impedance spectroscopy (EIS) designed for measuring antibody-rRAP-1/CT-STR antigen interactions, as described further.

3.2. Developing of the rRAP-1/CT-STR EIS

3.2.1. Formation of the sensing layer

EIS using antigen-sensitized gold electrodes were successfully used as a sensitive method to probe the interface properties of surface modified electrodes. Previous work has demonstrated the feasibility of this technique for a direct sensing of immunoreactions

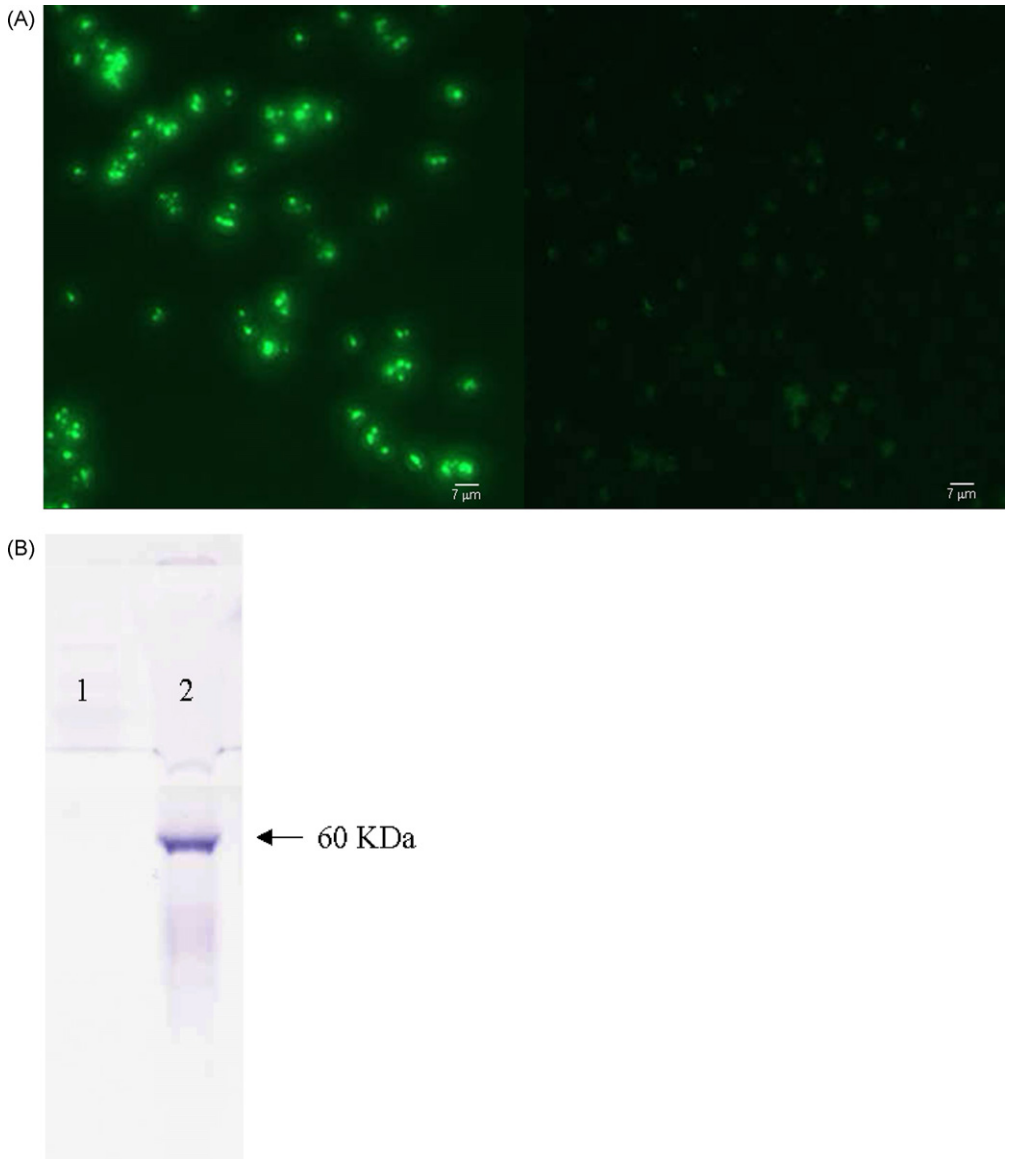

Fig. 1. Characterization of rRAP-1/CT-STR: (A) mouse polyclonal anti-rRAP-1/CT-STR antibody in *B. bovis* Mo7 strain recognized a parasite in an infected erythrocyte (left-side); non-infected erythrocyte as a negative control (right side), with 630× amplification. (B) Mouse polyclonal anti-rRAP-1/CT-STR antibody specifically recognized a 60 KDa band in *B. bovis* Mo7 strain in western blot analysis. Non-infected erythrocyte membrane (lane 1), infected erythrocytes (lane 2).

[25,26]. During the preparation of the gold electrodes each modification step was controlled with EIS. The measurements were done in PBS redox.

The formation of SAMs of sulphur-containing compounds onto the gold substrates is based on the strong interaction between gold-sulphur, which in the case of thiols has been calculated to be >150 hJ mol^{-1} [27]. Due to this interaction, SAMs of thioctic acid can be successfully formed through the oxidation of the linkage sulphur and hydrogen with the gold surface and the reduction of hydrogen at ambient conditions. The complex impedance is displayed as the sum of the real (Z_{re}) and imaginary (Z_{im}) components. Fig. 2 shows the impedance responses (cross lines) of the bare gold electrode (a), the SAM modified gold electrode after activation with EDC/NHS (b), the rRAP-1/CT-STR *B. bovis* protein, immobilized on gold electrode (c), and casein layer, the protein used to block the free spaces on gold electrode (d) in the frequency range from 100 kHz to 50 mHz.

The impedance spectra could be interpreted by the equivalent circuit mode, schematically displayed in Fig. 3. The fittings of each measured spectrum to the equivalent circuit are shown in Fig. 2 (solid line) indicating good agreement between the circuit model and the measurement system. The circuit includes ohmic resistance representing the electrolyte solution, R_S; a Warburg impedance, W, which is a consequence of the ions diffusion from the bulk of the electrolyte to the electrode interface; the impedance Z_{CPE} which is the constant phase element (CPE) impedance indicating the depressed semi-circle obtained; and an electron transfer resistance, R_{ct}. Ideally, W and R_S represent the properties of the electrolyte solution and the diffusion of the PBS redox probe, thus they are not affected by modifications occurring on the electrode surface. On the other hand, CPE and R_S are related to the dielectric and insulating features at the electrode/electrolyte interface, hence they are affected by the changes at the electrode surface. The results of the experimental data fitting using the equivalent circuit are summarized in Table 1. As can be seen in Table 1, there is some discrepancy between the experimental values (Fig. 3) and the simulated values. This discrepancy is caused by lack of a perfect semi-circle (likely due to non-homogeneities of grafted layers, defect area, surface roughness, etc.), and because our model is based

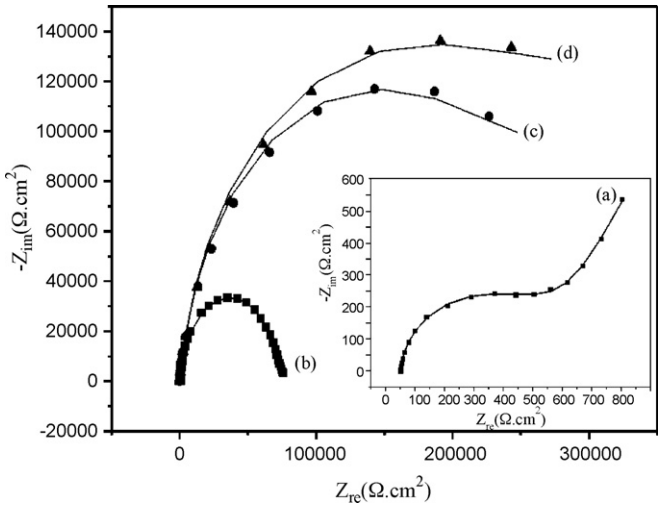

Fig. 2. Nyquist diagram (Z_{re} vs. Z_{im}) for impedance measurements corresponding to: (a) bare gold electrode; (b) SAM modified gold electrode after activation with EDC/NHS; (c) rRAP-1/CT-STR *B. bovis* protein/SAM/gold electrode; (d) casein/rRAP-1/CT-STR *B. bovis* protein/SAM/gold electrode. Solid curves show the computer fitting of the data using the equivalent circuit shown in Fig. 3. Symbols show the experimental data.

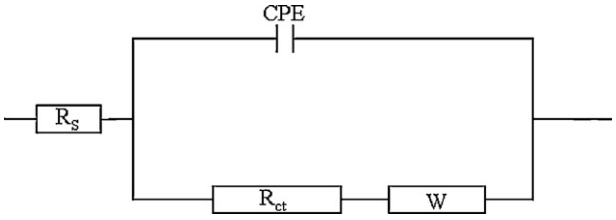

Fig. 3. Diagram showing the equivalent circuit for the impedance spectroscopy measurement.

on the CPE method, where we replace the impedance with the CPE element [24,25,26].

3.2.2. Detection of antibodies against B. bovis rRAP-1/CT-STR protein by EIS

B. bovis rRAP-1/CT-STR protein immobilized on the gold layer was attached to the electrochemical cell wall and different concentrations of mouse polyclonal anti-rRAP-1/CT-STR antibody were added at room temperature, into the electrochemical cell. The initial concentration of antibody in the polyclonal anti-rRAP-1/CT-STR serum was estimated by comparison with a standard calibration curve using known amounts of monoclonal antibody BABB75 in an ELISA format as described in Section 2.7 [14]. The impedance spectrum was measured for each calculated concentration of polyclonal antibody added, and after the impedance stabilization was achieved, a new concentration of antibody was added to the flow cell. Fig. 4A shows the impedance spectra obtained after stabilization for different concentrations of antibody. Since the impedance spectra obtained increases in a linear fashion with increasing concentrations of anti-rRAP-1/CT-STR antibody, ranging from 0 μM

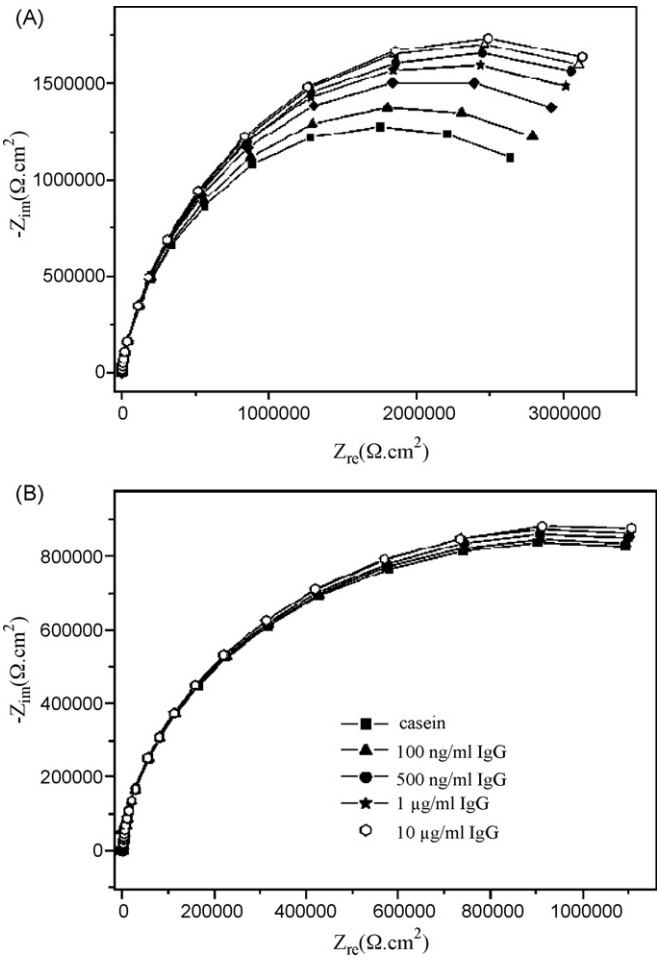

Fig. 4. (A) Complex impedance plots for impedance measurements in the presence of 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ for the immunosensor after incubation with solutions of 0 μM (solid square), 16.7 μM (solid triangle), 83.3 μM (solid rhomboid), 167 μM (solid star), 500 μM (solid circle), 2.5 mM (clear triangle) and 5 mM (clear circle) anti-rRAP-1/CT-STR *B. bovis* antibody. Solid curves show the computer fitting of the data using the equivalent circuit shown in Fig. 3. Symbols show the experimental data. (B) Nyquist plot for impedance measurements for anti-rabbit IgG antibody.

to 5 mM, this results in a detection range between 16.7 and 500 μM. An observed increase in the semicircle diameter in the Nyquist plot associated with an increase in the anti-rRAP-1/CT-STR antibody implies that more antibodies are being bound to the interface as the antibody concentration increased, thus resulting in a large interfacial R_{ct} (charge transfer resistance). When the concentration of antibody was increased over 500 μM, the change of impedance spectroscopy gradually levelled out, showing that the immobilization of the protein onto the gold electrode tends toward saturation. All the impedance spectra were fitted to the equivalent circuit in order to obtain the values of charge transfer resistance at each concentration. In order to compare the different electrodes' measurements in equivalent conditions, the relative val-

Table 1
The fitting values of the equivalent circuit elements for stepwise formation of the multilayer films by Zview software.

	R_S ($\Omega\,cm^2$)	CPE ($\mu F\,cm^{-2}$)	α	R_{ct} ($\Omega\,cm^2$)	W ($\Omega\,cm^2$)
Bare gold	29.5	3.36	0.942	550	3561
Thiol SAM activated	26.71	4.05	0.938	73400	–
rRAP-1/CT-STR protein	24.83	4.46	0.912	2.29×10^5	160
Casein	24.79	4.55	0.909	2.41×10^5	307

ues $(R_{ct(i)} - R_{ct(0)})/R_{ct(0)}$ were used. $R_{ct(0)}$ and $R_{ct(i)}$ are respectively the charge transfer resistance of the rRAP-1/CT-STR protein layer before and after reaction with antibody.

An additional control was added in order to check if there is any non-specific binding to the gold surface. For that, a bovine anti-rabbit IgG was injected into the flowcell and incubated for 60 min at room temperature. No significant variation of capacitance was measured in EIS when using this control antibody (Fig. 4B).

3.3. Detection of antibodies against B. bovis rRAP-1/CT-STR protein by EIS in serum from field cattle

The EIS is able to detect mouse polyclonal antibodies against anti-rRAP-1/CT-STR, as shown in Section 3.2.2. To determine whether an EIS-based system is also able to detect antibodies reactive with rRAP-1/CT-STR present in serum samples obtained from cattle in the field, we compared four selected serum samples that were also analyzed by ELISA and IFAT. The four serum samples were chosen according to their pattern of reaction with *Babesia* antigens using distinct serological diagnostic assays. Thus, bovine sample numbers 1 and 2 were both found positive for interaction with *B. bovis* antigens when using both, IFAT and ELISA techniques. However, bovine sample number 1 had a strong positive reaction to *B. bovis* antigens in both procedures, whereas sample number 2 also showed a positive but weaker reaction. As shown Table 2, two additional bovine serum samples (samples 3 and 4) that had given ambiguous results using these two techniques were also included for testing by EIS. Results of these comparisons are shown in Table 2. Both, ELISA and EIS showed identical results, with three positive and one negative cattle serum samples. In contrast, the IFAT method is in disagreement with the ELISA and EIS tests for two out of the four samples tested (Table 2). These results are not unexpected given the subjective nature of the IFAT test [4,7], and the fact that both, ELISA and EIS were designed for detecting only specific antibodies against the rRAP-1/CT-STR antigen. Therefore, although the results suggest that EIS could also be used as a method for the detection of antibodies in cattle samples using *B. bovis* rRAP-1/CT-STR protein immobilized on gold substrate, more field cattle serum samples needs to be analysed in future work comparing these three antibody detection methods using an statistically validated design.

In addition, and to gain information on the sensitivity of the EIS, we analyzed different increasing dilutions for each of the bovine serum samples analyzed. These comparisons showed that saturation of the gold electrode occurred at the lowest dilution (1:12.5), whereas using dilutions higher to 1:250 did not result in any measurable reaction against the rRAP-1/CT-STR antigen.

3.4. Evaluation of the association constant of antibody to B. bovis rRAP-1/CT-STR using EIS

Monitoring bio-molecular interactions plays a crucial role in biomedical or diagnostic field. Several labelling techniques have been established for this purpose: fluorescence, luminescence and radioactive labelling. However, the labelling of bio-molecules such as proteins and antibodies can be disadvantageous, especially if the label occupies an important binding site and thus influences the binding process leading to false information about the rate constants of interaction [28]. In contrast, EIS allows monitoring the antibody–protein interaction in real time without labelling of molecules. The antibody–antigen association constant was calculated by using a Langmuir isotherm. This approach has been successfully applied previously for determination of binding constants between cations and crown ethers [27,29].

In the experiment presented here, the changes of R_{ct} were related to the binding of rRAP-1/CT-STR protein to anti-rRAP-1/CT-STR antibody from *B. bovis*. The relation between the occupied binding sites Θ and the change of R_{ct} is as follows [30]:

$$\Theta = 1 - \frac{R_{ct(0)}}{R_{ct(i)}} \quad (1)$$

In the case of Langmuir isotherm, Θ can be related to the association constant according to the equation [27]:

$$\Theta = \frac{K_a c}{1 + K_a c} \quad (2)$$

where K_a denotes association constant and c is the concentration of molecules in the solution.

The linearization of Langmuir isotherm gives:

$$K_a c = \frac{\Theta}{1 - \Theta} \quad (3)$$

Eqs. (1) and (3) give:

$$K_a c = \frac{R_{ct(i)} - R_{ct(0)}}{R_{ct(0)}} \quad (4)$$

Eq. (4) has been applied to the calculation of the association constant for the *B. bovis* rRAP-1/CT-STR antibody–protein complexes.

The $R_{ct(i)}$ varies linearly with the concentration of antibody in the concentration range 16.7–500 μM as seen in Fig. 5.

As mentioned previously in Section 3.2.2, we used a polyclonal antibody to perform some of these assays. Considering the effect of the affinity heterogeneity in antiserum, it would be more appropriate to characterize the antibody population by defining an average, rather than an individual association constant. This average association constant could then be calculated from the slope of the $(R_{ct(i)} - R_{ct(0)})/R_{ct(0)}$ ratio versus the concentration. Thus, using this approach, the association constant value obtained for the interaction between rRAP-1/CT-STR and the anti-rRAP-1/CT-STR mice

Table 2
Detection of antibodies against *B. bovis* in four selected cattle serum samples by ELISA, IFAT and EIS.

Methods	Serum number 1	Serum number 2	Serum number 3	Serum number 4
ELISA	Positive	Positive	Positive	Negative
IFAT	Positive	Positive	Negative	Positive
EIS	Positive	Positive	Positive	Negative

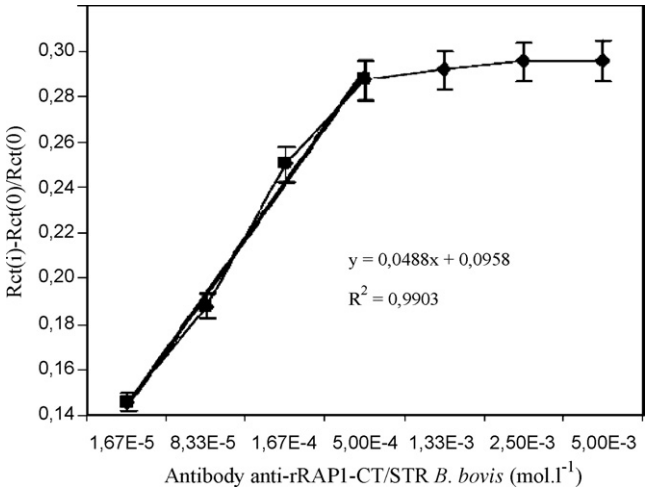

Fig. 5. Linear relationship of the $(R_{ct(i)} - R_{ct(0)})/R_{ct(0)}$ versus concentration of anti-rRAP-1/CT-STR antibody.

antibody was 48.8×10^{-3} M. The association constant is an important measurement in biological assays as a parameter to asses the affinity of interactions. Association constant values are used to characterize protein–protein and protein–DNA interactions, studies of immunoreactions, characterization of immune responses, detection and quantification of synthesized proteins, monitoring of enzyme kinetics of drugs and for label free primary and secondary screening for small compounds, antibodies or peptides in active component search [31].

4. Conclusion

The present study employs for the first time an impedance spectroscopy immunosensor (EIS) for the characterization of the interaction between antigen and antibodies against *B. bovis* proteins. The EIS device uses a recombinant version of the RAP-1/CT antigen cloned from a Portuguese *B. bovis* strain termed Santarém (rRAP-1/CT-STR). The alteration of the interfacial properties of the electrodes upon protein–antibody interaction was successfully traced by EIS technique.

The charge transfer resistance of the casein/rRAP-1/CT-STR protein/thioctic acid complex deposited onto a gold electrode surface changes linearly with the concentration of the anti-rRAP-1/CT-STR antibody in the range estimated between 16.7 and 500 μM. The EIS was tested with a small number of positive and negative sera of naturally infected animals, showing agreement with the standard ELISA and IFAT methods. These preliminary results suggest that further development of a biosensor for detecting antibodies against *B. bovis* antigens based on impedance measurements could be pursued. However, further field studies should be performed using a much larger number of field samples for determining the diagnostic value of the test. The EIS developed here also presents an advantage over standard immunoassay techniques since it is a label free technique thus allowing monitoring of antibody–antigen interactions in real time, without changing their properties. In addition the EIS provides an understanding of the dynamics of bio-recognition events by the determination of an association constant, an important measurement in biological assays in areas such as *in vitro* neutralization studies and cell adhesion [31].

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